Factors affecting formation of large calcite crystals (≥1mm) in ***Bacillus subtilis*** 168 biofilm

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Abstract

B4 is the most common medium used in general organomineralization studies and has been used to assay or to characterize mineral precipitation potential. In an exercise for the optimization of the laboratory conditions of crystal precipitation *in vitro*, we used *Bacillus subtilis* 168 as a type strain and its isogenic mutants. While literature is mainly focused on observing generic precipitation, we investigated the requirement to obtain large crystals (≥1mm), which could be advantageous in wide-ranging implications for bioconsolidation of soil, sand, stone, and cementitious materials. Calcite crystals are visible on B4 agar plates within 7 days at 37˚C after inoculum of *B. subtilis* 168 strain. In this study we show that to form large crystals with a diameter ≥1mm several conditions must be met: i) Reduced amount of B4 medium into the Petri plate improve crystal formation. 55mm Petri plates contained only 4mL of B4 agar medium reached a plateau in 6 days at 37ºC. High moisture and presence of water condense would decrease crystal formation. ii) Inoculation of cells using a rod instead of a circular shaped spot. When the same number of *B. subtilis* cells was streaked, rod-shape biofilm significantly fostered crystal precipitation, while spot-shape prevented precipitation. iii) When more than one biofilm is present within the same plate,

mutual interactions can affect precipitation in each biofilm. iv) Spherical nucleation sites are identified as initial step during the formation of large calcite crystal.

Keywords: Biofilm formation, *Bacillus subtilis*, calcite crystals, $CaCO_3$, organomineralization, B4

Introduction

B4 is extensively used to assess potential mineral precipitation on biofilm *in vitro*. We used *Bacillus subtilis* 168 as a type strain and its isogenic mutants as model organism to study calcite organomineralization on bacterial biofilms while growing on it. No work is currently focusing to the size of the crystal *in vitro* conditions. The investigation of the requirement to obtain large crystals (≥1mm) could be advantageous in wide-ranging implications for the bioconsolidation of soil, sand, stone, and cementitious materials. Recently, bacterial biomineralization has drawn the attention for its importance in many fields. It is relevant in ecology: $CaCO_3$ precipitation in hot springs (Jones and Peng 2014), fresh water (Saunders et al. 2014) and marine cyanobacterial mats (Kazmierczak et al. 2015) to cite some examples. From a more practical stand point, $CaCO_3$ mineral precipitation is involved in innovative technologies for stone consolidation, for example, the generation of inorganic material on bed biofilm reactor-membrane bioreactors (Gonzalez-Martinez et al. 2015), and as a new tool in the conservation of monumental calcareous stones (Perito et al. 2014; Zamarreño et al. 2009).

For these applied subjects, the need for a laboratory *in vitro* model system is required and also the size of the crystal is important. Previous studies have already used *Bacillus subtilis* 168 as a type strain to study calcite precipitation on biofilms by using the maximum medium B4 rich in calcium (Barabesi et al. 2007; Marvasi et al. 2010; Marvasi et al. 2012; Boquet et al. 1973). Calcite crystals are obtained by streaking *B. subtilis* 168 strain on B4 medium and calcite crystals are visible on B4 within 7 days at 37˚C.

Furthermore, we have previously isolated isogenic *B. subtilis* 168 mutants unable to precipitate calcite crystals on B4 (Barabesi et al. 2007). The mutated genes belong to the *lcfA* operon, with functions involved in fatty acid metabolism. Our data pointed to an involvement of the whole cluster in the precipitation phenotype and suggested a link between calcite precipitation and fatty acid metabolism. We hypothesized possible pleiotropic effects of mutations in fatty acid pathways on cellular metabolism or *lcfA*

operon could be involved in the synthesis of a lipid intermediate (e.g. an acyl CoA intermediate) directly involved in biomineralization (Barabesi et al. 2007, Tojo et al. 2011; Surorova et al. 2015). In a following study we demonstrated that the decrease in pH of the FBC5 biofilm, the strain carrying a mutation in last gene of the lcfA operon, *etfA*, was the main process responsible for the impairment in precipitation. Since EtfA is known to be involved in redox reactions of NAD+/NADH, we proposed that a misregulation in some part of the EtfA pathway could be responsible for NADH accumulation in FBC5 and consequently for exceeding extrusion of H^+ (Marvasi et al. 2010).

Calcium carbonate deposition by bacteria is largely dependent on environmental conditions (Wei et al. 2015; Pedley 2014; Beveridge 1989; Perito and Mastromei 2011). On B4, precipitation is controlled by factors such as the pH of the medium and the acidification released by the biofilm during metabolic processes including carbonic acids and extrusion of protons via respiration chain (Marvasi et al. 2010). In this sense *in vitro* studies may help to compare carbonate structures typical isolated from the environment. For example spherical shapes (typical of spherulites or leiolites at macroscale), which originate from a combination of extracellular polymeric substances (EPS) and chemical crystallization, have been observed in the environment (Dupraz et al 2009a; Wei et al. 2015; Shirakawa et al. 2011; Banerjee and Joshi 2014). For example in freshwater microbial deposits often show carbonate precipitation on impregnation of cyanobacterial sheaths or cells. Likewise, spherulites (calcitic fibro-radial spherulitic poly- crystals) in subaerial calcrete laminar crusts are also a product of photosynthetically induced calcium carbonate precipitation (Verrecchia et al, 1995; Dupraz et al 2009a). We were intrigued whether the formation of large crystals would have let the observation of such shapes in the *B. subtilis* biofilm – in a not photosynthetic environment.

In this study we show that to form large crystals with a diameter ≥1mm several conditions must be met: i) Low water content; ii) Inoculation methods, iii) Mutual interactions of biofilms co-inoculated in the same plate. Finally, spherical nucleation sites have been observed during the formation of large calcite crystal.

Materials and Methods

Bacterial strains and B4 precipitation medium. Strains used in this study were *Bacillus subtilis* 168 (Anagnostopoulos and Spizizen 1961) and *B. subtilis* mutants described in the Supplementary Material Table S1 (Barabesi et al. 2007). To test calcite crystal formation the cultures were routinely grown on B4 solid (0.4% yeast extract, 0.5% dextrose, 0.25% calcium acetate, 1.4% agar) (Boquet et al. 1973). Calcite was identified via energy-dispersive X-ray spectroscopy (Barabesi et al. 2007). Mutant strains were supplemented with chloramphenicol 5 mg/ml (SIGMA) to maintain the selective pressure for maintaining the inserted plasmid. Unless otherwise specified, biofilms were grown on plates incubated at 37ºC inside a plastic bag to prevent dehydration. Bromothymol blue (SIGMA) which transition of pH ranges from yellow pH 6.0, green pH 6.7 and blue pH 7.6 was used as a pH indicator. Bromothymol blue stock solutions (20 mg/ml in NaOH 0.1N) were added to B4 medium before autoclaving at a final concentration of 0.0025% (v/v).

Test for calcium carbonate precipitation (in-vitro). Petri dishes 55mm (Fisher) were filled with 4 mL or 10 mL B4 agar medium according with the experiment. All strains were streaked as a single 4-cm streak at the center of the B4 agar plate by using a volume of 50µL of 10^8 CFU/mL from an overnight culture of *B. subtilis* in LB (Oxoid). Standard incubation occurred by putting plates into a plastic bag to prevent dehydration during the 14 days of incubation at 37°C. When additional moisture was requested, a 2x2cm filter paper (Whatman) soaked with 2 mL of sterile water was placed on the lid during the incubation of the B4 agar plates. B4 plates inoculated with *B. subtilis* 168 were daily observed to measure crystal precipitation. Five replicas for each experiment were performed. We were interested in the formation of large and visible calcite crystals on the biofilm. To that end only crystals with a diameter larger than ≥1mm were counted. According with the formation of single, isolated crystals on the biofilm, the following numerical score was associated: (0) no crystal formation; (1) First single crystal (≥1mm) was observed; (2) Between 2 and 10 crystals (≥1mm) were counted; (3) More than 10 crystals (≥1mm) were counted. Observations occurred visually by using a stereomicroscope (Olympus SZ51). Pictures of the streaks were taken by using an Olympus camera mounted on the stereomicroscope (Olympus SZ51).

Diffusible extracellular factor assays. Two streaks of different *B. subtilis* 168 isogenic pairs (Supplemental

Material Table SI) were inoculated with an L shape with one arm of the L parallel and adjacent to the other strain (but not touching the other strain). The other arm of the L shape was distal. Distal arms were used as controls (Barber et al. 1997). In control plates a portion of agar medium was removed with a sterile scalpel to generate a void between the two parallel strains, this prevented passage of diffusible factors (and possible acidification or alkalization effects) between biofilms (Supplementary Material Figure S1). Plates were incubated at 37°C for 15 days.

Crystals desiccation. Biofilms were grown until well-developed calcite crystals were formed, crystals were desiccated at 40° C for 6 days. Pictures of crystal development in the biofilms according to the inoculation mode used were taken by using an Olympus camera mounted on the stereomicroscope (Olympus SZ51).

Scanning electron microscope (SEM) images. Crystals morphology was observed in fresh samples by electron microscopy (ESEM Quanta-200 FEI) without fixation.

Statistical analysis: JMP (SAS) statistical software was used to infer the *t*-test.

Results

In the course of the study, a number of conditions were considered as essential for large (≥1mm) *in vitro* crystal formation on *Bacillus subtilis* biofilms. The conditions are reported in the following paragraphs.

1. Moisture and total volume of B4 affect calcite crystal formation on B4 agar medium.

In order to investigate to what extent moisture during biofilm development impaired calcite crystals formation, 55mm Petri plates with different B4 agar content were prepared. Biofilms (4 cm long) of *B. subtilis* 168 were streaked on B4 plates filled with 10mL of B4 agar and crystals formation was recorded for 14 days as described in material and methods. Crystal formation was completed at day 12 (Figure 1, panel A). To measure whether the volume of B4 agar inside the plates contributed to calcite precipitation, plates with only 4 mL of B4 agar medium were also inoculated. Interestingly, plates containing 4 mL instead of 10 mL of precipitation medium reached a plateau in 6 days (Figure 1, Panel A).

To determine if an excess of humidity during incubation affected calcite precipitation, 10 mL plates with a filter paper soaked with water was placed inside the lid and plates were incubated face-down as usual. Under these conditions no crystal formation was observed (Fig. 1, Panel B). However, when the amount of B4 agar medium in the plates was reduced to 4 mL, precipitation was completed after 9 days of incubation (Fig 1, Panel B).

2. Rod-streaking shape and initial number of cell seem to favor precipitation of large crystals onto precipitation media.

We tested to what extent the initial number of cells and inoculation method (spotting vs streaking) affected calcite precipitation on B4 media. 50 µL of a 10^9 CFU/mL *B. subtilis* 168 suspension and relative dilutions (10^7 and 10^5 CFU/mL) were streaked as a rod-shape (4 cm long rod streak) or spotted (1.5 cm of diameter) (Figure 2, A and B) onto B4 agar plates. No significant differences were reported for any dilutions on the streak, however the type of plating – streak versus spot – significantly affected calcite precipitation (Figure 2). Interestingly, when cells were plated as spot, complete precipitation (≥10 crystals on the biofilm) was never observed (Figure 2, B). An overall reduction of calcite precipitation was measured when spots were compared with streaks. Significant increase in precipitation was observed on the spot where 10^5 CFU/mL were used when compared with suspensions with higher cellular concentrations (Figure 2, panel B, days 12 and 13). At the end of the 14-day test, cellular concentrations were not significantly different. Visual differences in precipitations may be appreciated in Figure 3, where a representative picture is shown (Figure 3, streak panel A and spot panel B).

3. Mutual interaction of biofilms contributes to calcite precipitation

To test to what extent *B. subtilis* biofilms are able to mutually affect each other for calcite precipitation, a diffusion test was performed (Barber et al. 1997). In this study we used strains of *B. subtilis* mutated in the genes of *lcfA* operon, a gene cluster with functions in fatty acid metabolism that we identified as involved in the precipitation phenotype in a previous study (Barabesi et al. 2007). Mutants used in this study are reported in Table I, many of them are not able to precipitate calcite crystals in their biofilms (Table I). Diffusion test was performed by streaking *B. subtilis* wild type and its isogenic form in an L-shape. Two

arms of the L are adjacent, while the distal arms are used as control and to test how far the diffusion proceeds. After 15 days of incubation, strains FBC2 and FBC3 coupled with *B. subtilis* 168 were able to restore the precipitation on parallel arm (Table I). When pairs of the same *B. subtilis* mutant impaired in calcite formation were tested, precipitation did not occur (Table I).

Strains FBC4 and FBC5 coupled with *B. subtilis* 168 were unable to precipitate calcite in standard B4 medium (Table I). In FBC3, diffusion test showed that the adjacent streak section was able to partially restore the precipitation (Figure 4). As control, a portion of agar in between the two parallel strains (168 and FBC3) was removed. Mutual interactions were not observed due to the lack of diffusion (Table II).

We previously demonstrated that pH was indeed a main determinant in mineral precipitation since buffering B4 medium re-established calcite formation in *B. subtilis* mutants (Marvasi et al. 2010). To determine if pH changes could affect calcite precipitation behavior of FBC3 when streaked together with 168, a bromothymol blue pH indicator was added to the B4 precipitation medium. The arm of FBC3 streak adjacent to that of the wild type 168 was less acidic than the distal arm, indicating that re-establishment of calcite formation in the mutant can be due to alkalization released by the wild type (Supplementary material S2).

4. Spherical crystals serve as nucleation sites on B. subtilis biofilms*.*

Even though crystal formation on *B. subtilis* biofilms has been extensively studied, only a few studies focused on the initial nucleation step(s) of crystal nucleation during growth on B4 precipitation medium. Nanospheres of calcite were previously identified at early stages in calcite precipitation microbial mats (Dupraz et al. 2004). We therefore hypothesized that if early nucleation occurred within the biofilm, spherical calcite structures would be identified within the biofilm depth. Spherical structures were indeed observed after biofilm removal (Figure 5, A). Interestingly, spherical structures were connected with the external part of the crystal, revealing a putative early stage of crystal formation (Figure 5, B). SEM images also revealed spherical structure beneath the flat crystal structure (Figure 5, C and D) indicating that as the potential origin of nucleation sites on *B. subtilis* biofilms.

Discussion

196 Due to the relevance of biologically induced mineralization we decided to study several physical
197 conditions required for calcite formation of large crystals (≥1mm in diameter) in *B. subtilis* by using B4 as
198 precipitating medium. Several factors were considered as essential for large crystal formation in *B. subtilis*
199 biofilm. First, water content (thickness of the medium, presence of moisture). Active carbonate nucleation
200 occurs when bacterial cell surfaces are utilized as nucleation sites, owing to the chemically heterogeneous
201 macromolecules that impart a net electronegative charge, which favors the adsorption of cations with
202 CO_3^{2-} or HCO_3^- (Schultze-Lam and Beveridge 1994). In this context water plays a pivotal role. The
203 equilibrium constant for the dissolution of $CaCO_3$ is K_{ps} $4.5x10^{-9}$ and when the ionic product $Q_{sp} > K_{sp}$ a
204 precipitate is expected to form (Blackman A et al. 2016). A reduced amount of water would push the
205 equilibrium of the reaction toward the formation of salt. High concentration of calcium will favor the
206 formation of calcite on biofilm, and indeed precipitation occurred faster and at higher rate where on the
207 plates that don't contain the paper soaked with water. It is reasonable to assume that a minor volume of
208 B4 (4 ml), and therefore water, would foster precipitation.
209 Water is not the only factor limiting crystal formation. Streak's shape curiously also affects crystal
210 formation. We found that spotting *B. subtilis* 168 cells on B4 resulted in a decreased and delayed
211 production of crystals when compared with the a 4-cm long streak. On the other hand, the number of cells
212 seems to affect precipitation in the spot, with diluted inoculum (10^5 CFU/mL) inducing a better precipitation
213 on day 12^{th} and 13^{th} (Figure 2, panel B). We can speculate on causes that can be responsible for the
214 reduced precipitation in spot: i) Higher number of cells per cm^2 in the spot compared with the streak due to
215 the dilution of cells along the 4 cm of the streak itself; ii) unequal total area covered by the spot and the
216 streak leading to difference given the resources available to the cells; iii) the differences seen in spotting
217 and longitudinal streaking might be a function of chemical concentration. A lateral (longitudinal) streak
218 would allow the agar near the middle of the streak to be exposed to a higher concentration of crystal-
219 inducing chemical environment, which would not be evident in a spot inoculation. iv) The physiology of the
220 biofilm at the edge. Spatiotemporal analysis showed differentiation within a biofilm with zones (Vlamakis et
221 al. 2008) where motile cells, and therefore more active in respiration, NAHD production are at the edges
222 ultimately resulting in an increase of acidity (Vlamakis et al. 2008).

This observation is not surprising since microorganisms can change the microenvironmental conditions with their metabolism (Marvasi et al. 2010; Marvasi et al. 2012; Dupraz et al. 2009b; Dupraz et al. 2008). Biologically induced biomineralization is characterized through a delicate equilibrium where the environment and the microbial metabolism play together to foster and shape crystals. In this study we have shown that biofilms can affect each other by mutual pH interactions by using a diffusion test. Results from the diffusion tests indicate that the alkalization of the medium induced by stronger alkalizing strains (such as *B. subtilis* 168), do foster crystal formation on the parallel section of the acidifying biofilm. However, strains previously reported to decrease the pH of their biofilms during growth (ie. strains FBC5 or FBC4 (Marvasi et al. 2010) prevented crystal formation and reduced precipitation in 168 biofilm (Table I). We cannot exclude that other molecules may be responsible for the restoration of the precipitation from strains capable of calcite precipitation to impaired mutant, however pH is currently the main responsible of the restored precipitation, since using media buffered at different pH values calcite formation was controlled (Marvasi et al. 2010). These data show it is necessary to streak one single bacterial strain per plate to observe real precipitation and prevent influences from other strains. Other factor that affects precipitation when two or more bacteria are closely grown is the depletion of calcium. Distinct strains can deplete different amount of calcium (Shirakawa et al. 2011). *Pseudomonas putida*, for example, consumed on average 96% of all Ca^{2+} available, *Lysinibacillus sphaericus* 74% and *Bacillus subtilis* only 28% calcium ion compared to the control of B4 without bacteria within 12 days of incubation. In addition, the ratio of different competing ions such as magnesium and calcium has been proved to affect the complexation with CO_3^{2-} (Mg/Ca) (Saunders et al. 2014). Experiments conducted from lithifying biofilm isolated from River Lathkill (UK) showed that the $(Mg/Ca)_{calcite}$ precipitation rate, rapidly formed precipitates with very low magnesium content indicating kinetic control on fractionation (Saunders et al. 2014). These data show as the type of biofilm and biofilm growth rate control calcite precipitation (Saunders et al. 2014). These results support, once more, the necessity to streak one strain per Petri plate when testing its calcinogenic potential.

Another interesting finding of this *in vitro* system was the presence of spherical crystals as nucleation sites on *B. subtilis* biofilms as reported in the SEM images. The initial nucleation may occur as a sphere and

when the crystal reaches the upper surfaces the shape changes by growing as a flat crystal. We recognize that this spherical shape may occur on *B. subtilis* 168 due to the presence of a defined extracellular polymeric substances (EPS) composition which affects crystal shape (Braissant et al. 2007). Such spherical shapes have been observed in different bacterial genera and species and the model may play a role in other biofilms and environments (Wei et al. 2015;Shirakawa et al. 2011;Banerjee and Joshi 2014).

In conclusion, we show that to form large crystals with a diameter ≥1mm several conditions must be met. Reduced amount of B4 medium in the Petri plate we recommend 55mm Petri plates contained only 4mL of B4 agar showed the best performance. Presence of water condense would decrease crystal formation. Inoculation of cells using a rod instead of inoculating a circular shaped spot. Mutual interactions can affect precipitation in each biofilm, so we recommend streak one biofilm per plate. This inoculation strategy will lead to the formation of spherical nucleation.

Further experiments of such experimental conditions could be used to make mathematical predictions regarding the geochemical conditions required to yield mineral precipitation, including crystal size.

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Captions

Table I. Results of diffusible factor assay.

Table II. Diffusion test on plates with agar removed.

Figure 1. Effect of moisture and volume of B4 medium on crystal formation. Panel A shows crystals growth on standard B4 medium. Panel B are plates in which contains a 2x2 cm filter paper soaked with water and incubated on the lid into face-down incubated 55mm Petri plates. Error bars represent the standard errors. Five replicas for each measurement were performed. The lines show a smooth curve through the data. It is an approximating function that attempts to capture the pattern of the data.

Figure 2. Crystal formation by spotting or streaking serial dilutions on *B. subtilis* 168 on B4 medium. Panel A, crystal formation of different dilutions of rod-shape biofilms. Panel B, crystal formation of serial dilution with a circular-shaped biofilm. Asterisk (*) shows significant difference ($p<0.05$). Error bars represent the standard errors. The lines show a smooth curve through the data. It is an approximating function that attempts to capture the pattern of the data.

Figure 3. Crystal formation on rod and spot shaped biofilm. *B. subtilis* cells were spotted with different shape resulting in different crystal precipitation. Panel A, B shows 50µL of 10^9 CFU mL^{-1}. Panels C, D are 50µL of 10^9 CFU mL^{-1}. Arrows show representative crystals with a diameter ≥ 1mm. In panel B only one crystal >1mm is identified. Each panel is made by recomposing a number of individual pictures taken with the stereomicroscope. Photos taken from biofilms grown on 90mm Petri plates.

Figure 4. Diffusion test of the pair *B. subtilis* 168/FBC3 after 7 days of incubation at 37ºC. Representative crystals with a diameter ≥ 0.8mm are shown with an arrow. Dotted bracket highlights an area with small calcite crystals.

Figure 5. Spherical nucleation sites of calcite crystals produced by *B. subtilis* 168 biofilm. Panel A, stereomicroscope image of calcite crystals. The black arrow shows a spherical nucleation site. Panel B, stereomicroscope image at higher magnification of a crystal with the characteristic “umbrella” shape. Panel C, ESEM micrograph of an agglomerate of calcite crystals produced by *B. subtilis* 168 biofilm. The micrograph shows the bottom faces of the agglomerate. White arrows show calcite spheroid nucleation sites. Panel D, Upper face. White arrows show flat circular area where crystals emerge from biofilm.

Supplemental Material Figure S1. The two strains are depicted by a dotted and continuous line. Gray rectangle represent agar removed for the control.

Supplemental Material Figure S2. Qualitatively evidence of pH gradient (diffusion). pH of B4 medium where strains 168 and FBC3 were streaked highlighted with the pH indicator bromothymol blue. In the picture the indicator is lightly gray (pH 6.0), and dark gray where the pH is 7.6 where the precipitation occurs. The distal arm of the mutant strain *B. subtilis* BCF3 is more acid (light gray in the picture) when compared with the arm adjacent to the wild type *B. subtilis* 168. Arrows indicate the differences in color.

Supplemental Material Table S I. Strains used in this study.

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